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NOTES

Double-Stranded RNA Adenosine Deaminase ADAR-1-Induced Hypermutated Genomes among Inactivated Seasonal Influenza and Live Attenuated Measles Virus Vaccines▽

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We sought to examine ADAR-1 editing of measles and influenza virus genomes derived from inactivated seasonal influenza and live attenuated measles virus vaccines grown on chicken cells as the culture substrate. Using highly sensitive 3DI-PCR (R. Suspène et al., Nucleic Acids Res. 36:e72, 2008), it was possible to show that ADAR-1 could hyperdeaminate adenosine residues in both measles virus and influenza virus A genomes. Detailed analysis of the dinucleotide editing context showed preferences for 5′ArA and 5′UrA, which is typical of editing in mammalian cells. The hyperedited mutant frequency, including genomes and antigenomes, was a log greater for influenza virus compared to measles virus, suggesting a greater sensitivity to restriction by ADAR-1.

The host cell restricts viral replication by a myriad of mechanisms, those induced by type I interferons (IFNs) being among the best known (25, 30). Both cytidine and adenosine polynucleotide deaminases are induced and can restrict viral replication (3, 5, 13, 18, 20, 27, 28). For humans, there are 11 genes encoding cytidine deaminases (APOBEC1, APOBEC2, APOBEC3A to -C, -3DE, -3F to -H, APOBEC4, and AID), of which 8 are functional on single-stranded DNA (ssDNA) and only 1 of which is functional on RNA. In contrast, three adenosine deaminases acting on RNA (ADAR-1, -2, and -3) are known, their substrate specificity being double-stranded RNA (dsRNA) (3, 5, 16, 17). Although ADAR-1, -2, and -3 are conserved in their C-terminal catalytic domain as well as in their double-stranded RNA-binding domains, only ADAR-1 and -2 have demonstrable enzymatic activity (2, 8, 9). They probably evolved from adenosine deaminases acting on tRNAs after the split between protozoa and metazoa (17).

While mammalian ADAR-1 and ADAR-2 are ubiquitously expressed in many tissues, ADAR-3 was limited to the nervous system (8, 22). Of the two ADAR-1 gene transcripts, ADAR-1L and -1S, only the former can be induced by IFN-α/β and -γ (25). ADAR-edited measles virus genomes were first described in cases of subacute sclerosing panencephalitis (SSPE), which is a rare chronic degenerative disease that occurs several years after measles infection (24, 33, 34). Although part of an antiviral response, hepatitis D satellite virus actually requires RNA editing by ADAR-1L as an essential part of its life cycle (7). Aside from these important examples, ADAR-edited RNA viral genomes have remained few and far between, being confined mainly to negative-stranded viruses such as influenza virus A, lymphocytic choriomeningitis virus, respiratory syncytial virus, and paramyxovirus (4, 6, 21, 29, 35). To overcome this rarity, we recently developed a PCR-based method, referred to as "3DI-PCR," that allows selective amplification of GC-rich DNA, notably ADAR-edited sequences (28). It was possible to show that ADAR-1L can strongly edit measles virus (MV) and attenuated Rift Valley fever virus in culture (28). Many commercially available vaccines have been produced by growth on chicken embryo fibroblasts, which express an ADAR-1-like protein (1, 15). The question asked was whether such viruses are restricted by adenosine deaminases, particularly influenza virus A. Here, we have analyzed the live attenuated pediatric measles-mumps-rubella virus (MMR) and the seasonal influenza virus vaccines.

Two different commercially MMR vaccines lots NC29620 and NC70980 (Sanofi-Pasteur) were bought from local pharmacies. Total RNA was recovered, and cDNA synthesis with random primers was performed. A fragment of the M gene of measles virus was amplified by a nested procedure called "3DI-PCR," as described in reference 28. 3DI-PCR products were recovered at a PCR denaturation temperature as low as 65.1°C for the NC29620 and NC70980 vaccines, compared to 65.7°C, corresponding to the negative-control, measles stock viruses from Vero cells which are defective for type I interferon induction (28) (Fig. 1A). The 3DI-PCR products recovered at

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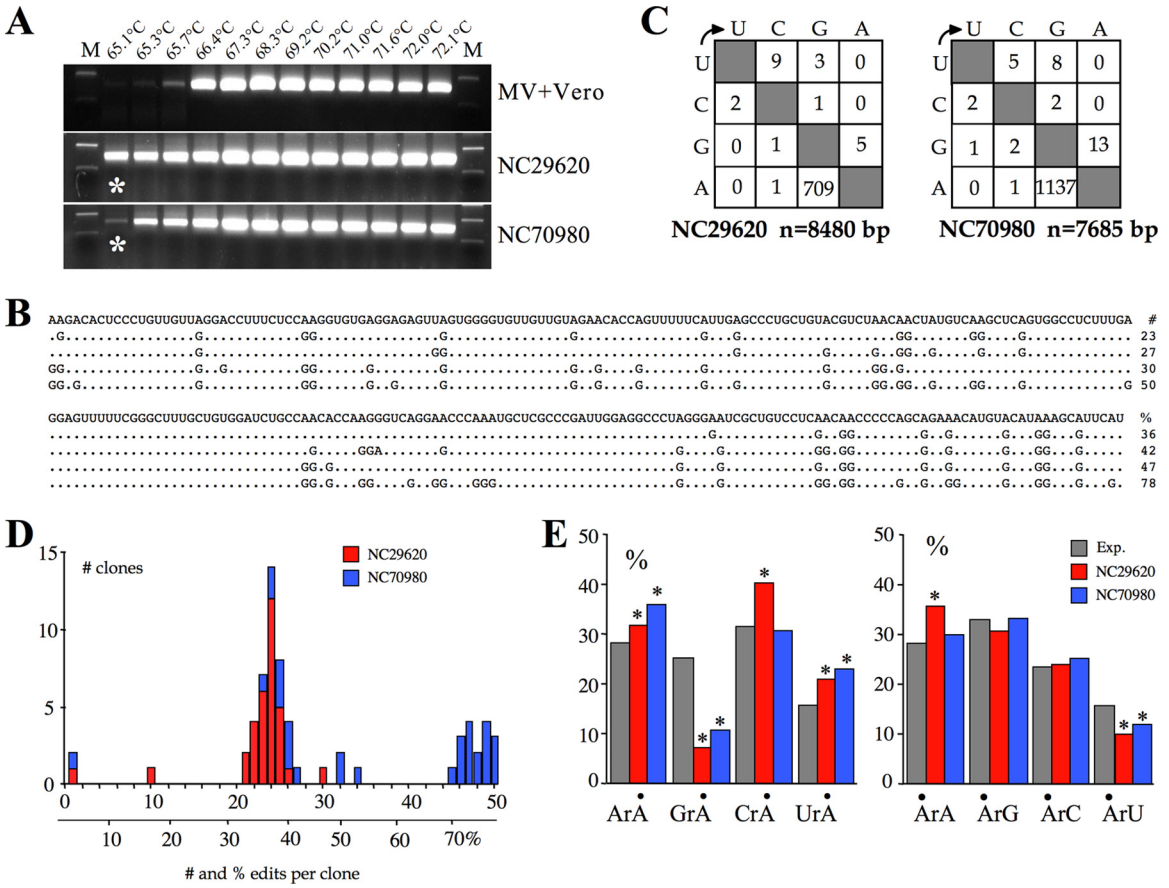

FIG. 1. ADAR editing of measles virus genomes from MMR vaccines. (A) 3DI-PCR of the measles virus M gene from two commercial MMR vaccine lots. The negative control was Vero cells infected by measles virus. M, molecular weight markers; MV, measles virus. Asterisks indicate the PCR products cloned and sequenced. The sizes of the 3DI-PCR fragments are 315 bp. (B) Example of measles virus hypermutated genomes. # and % indicate the number of A→G transitions and the percentage of A targets edited to G, respectively. (C) Mutation matrices of NC29620 and NC70980 hyperedited measles virus sequences. The numbers below the matrices indicate the number of bases sequenced. (D) Frequency distribution of A→G editing per clone. (E) Dinucleotide analysis in 5′ and 3′ of NC29620 and NC70980 edited measles genomes. The dot indicates the edited base. Exp., expected. A χ^2 analysis indicates dinucleotide frequencies deviating significantly from the expected values (*, $P < 0.05$).

65.1°C from both vaccine lots were cloned and sequenced. As can be seen, A→G transitions dominated (Fig. 1B and C). The mean editing frequencies for NC29620 and NC70980 were 35% (range, 2 to 47%) and 55% (range, 2 to 78%), respectively. These differences, which could reflect different ADAR-1L levels, are more obvious when hypermutant frequency spectra are compared (Fig. 1D). That for lot NC29620 is highly focused around ~37% editing, while that for NC70980 is more dispersed, albeit with a strong cluster around >70% ADAR editing. The dearth of lightly edited genomes may reflect negative selection by the 3DI-PCR method, a finding identified by using the sister technique (i.e., 3D-PCR amplification of AT-rich DNA) (31). The dinucleotide contexts associated with adenosine editing were similar for the two vaccine lots, with a slight preference for 5′ArA and 5′UrA and a strong aversion for 5′GrA (Fig. 1E), which agrees with the literature (19). No obvious 3′ context was observed (Fig. 1E).

In view of these findings, we decided to analyze an inactivated seasonal influenza virus vaccine produced by using chicken embryo fibroblasts, especially as there are no reports of strong ADAR editing of influenza virus genomes. These vaccines were composites of the A/New Caledonia/20/99 H1N1, A/Wisconsin/67/2005 H3N2, and B/Malaysia/2506/2004 strains (Sanofi-Pasteur lots A5807-2 and A0667-1), again bought from local pharmacies. A fragment of the hemagglutinin (HA) gene of influenza virus A H1N1 was amplified by a classic nested procedure. The first-round primers for influenza virus amplification were FNCout and RNCout (5′ CCCGCC YAACAYAGGGGACCAAAG and 5′ TCTGCAGCRTRGC CRGRTCCTTG, respectively), while the second-round primers were FNCin and RNCin (5′ AAAGYAAGAGAYCAGG AAGGAAG and 5′ GGAGGGRTGTTCCTTRGTCCTG, respectively). The PCR and 3DI-PCR protocols were similar to the MMR vaccine amplification. For the 3DI-PCR amplifications, the following conditions were used: 65 to 70°C for 5 min; followed by 35 cycles of 65 to 70°C for 30 s, 45°C for 30 s, and 65 to 70°C for 1 min; and finally 10 min at 65°C. PCR products were then purified and cloned in TOPO TA vector, as previously described, and between 30 and 80 clones were sequenced.

3DI-PCR products were obtained at the restricting temperature of 66.2 and 65.8°C (Fig. 2A). The products were cloned

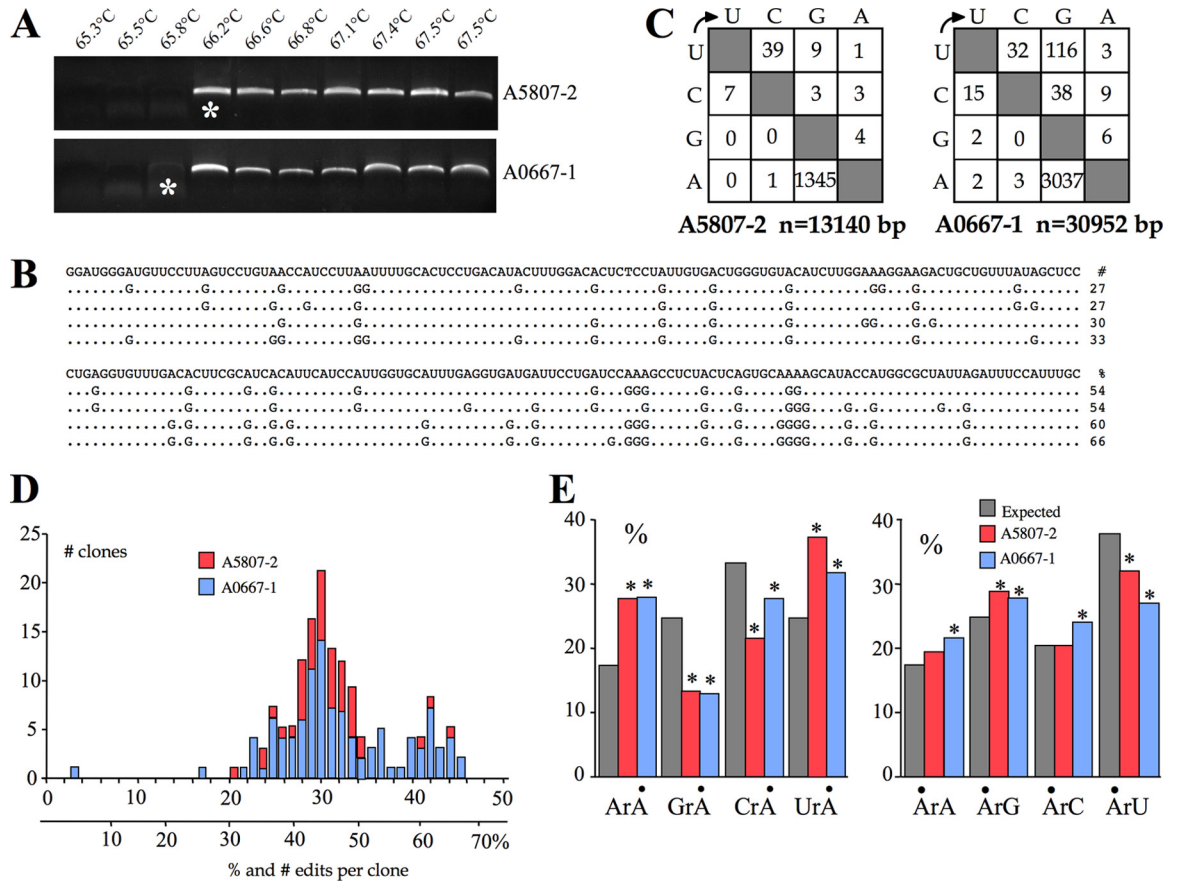

FIG. 2. ADAR editing of influenza virus A genomes from seasonal influenza virus vaccines. (A) 3DI-PCR of influenza virus A H1N1 vaccines (A5807-2 and A0667-1). Asterisks indicate the PCR products cloned and sequenced. The sizes of the 3DI-PCR fragments are 337 bp. (B) Some examples of hypermutated influenza virus genomes. (C) Mutation matrices of two hyperedited influenza virus sequences. The numbers below the matrices indicate the number of bases sequenced. (D) Frequency distribution of A→G editing per clone for the two vaccine lots. (E) Dinucleotide analysis in 5′ and 3′ of two edited influenza virus genomes. A χ^2 analysis indicates dinucleotide frequencies deviating significantly from the expected values (*, $P < 0.05$).

and sequenced. Hyperediting was essentially confined to the minus RNA strands (Fig. 2B and C). The mean adenosine editing frequencies for the two lots were comparable (A0667-1, mean of 47% and range of 4 to 66%; A5807-2, mean of 44% and range of 30 to 64%) (Fig. 2D). The dinucleotide context associated with adenosine editing was 5′ArA and 5′UrA sites, with no obvious context for the 3′ nucleotide (Fig. 2E).

The nonstructural influenza virus NS1 protein has been described to interact with ADAR-1 (10, 23). This interaction antagonizes the cellular IFN response, and viruses lacking the NS1 gene only replicate efficiently on an IFN-α/β-deficient background (32). To explore this experimentally, avian QT6 cells (14) were infected with the A/PR/8/34 strain and compared to its ΔNS1 counterpart, which encodes a 472-bp deletion (12). Briefly, 3×10^6 QT6 cells were infected, respectively, with the A/PR/8/34 and A/PR/8/34/ΔNS1 viruses. At 48 h postinfection, supernatant virus was harvested and total RNA was extracted. PCR and 3DI-PCR were performed under the same experimental conditions described above, with the denaturation temperature gradient ranging from 70°C to 64°C. A nested procedure was used to amplify a different HA fragment. The external primers were RPRout (5′ RTRGCCTGRTCCC TGTTCRTTCTG) and FPRout (5′ AYYCAYCACCCGYCY AACAGYAAG), while the internal primers were FPRin (5′ GAAAGACCCAAAGYAAGAGAYCAAG) and RPRin (G GRCGGRRTGTTCCTTRGTCCTG). At a denaturation temperature of 67.6°C, more DNA was recovered from the A/PR/8/34/ΔNS1 strain than from the wild-type control (Fig. 3A). These 3DI-PCR products were cloned and sequenced; a selection of hyperedited sequences is shown in Fig. 3B. As expected, most of the mutations were A→G transitions, with a mean adenosine substitution frequency of around 31% per clone (range, 7 to 60%) (Fig. 3B and C). A strong and significance preference was observed for 5′ArA and 5′UrA, which is similar to the dinucleotide context analyzed for the two influenza virus vaccines (Fig. 2E and 3D). Hence, the majority of edited sequences can be ascribed to the avian ADAR-1L deaminase.

In order to determine the overall editing frequencies in the attenuated viral vaccines, we performed deep sequencing on standard reverse transcription-PCR (RT-PCR) products on MMR (NC70980) and influenza virus (A0667-1) vaccines. In order to generate more sequence information, the PCR fragments were larger than those used for the 3DI-PCR analyses.

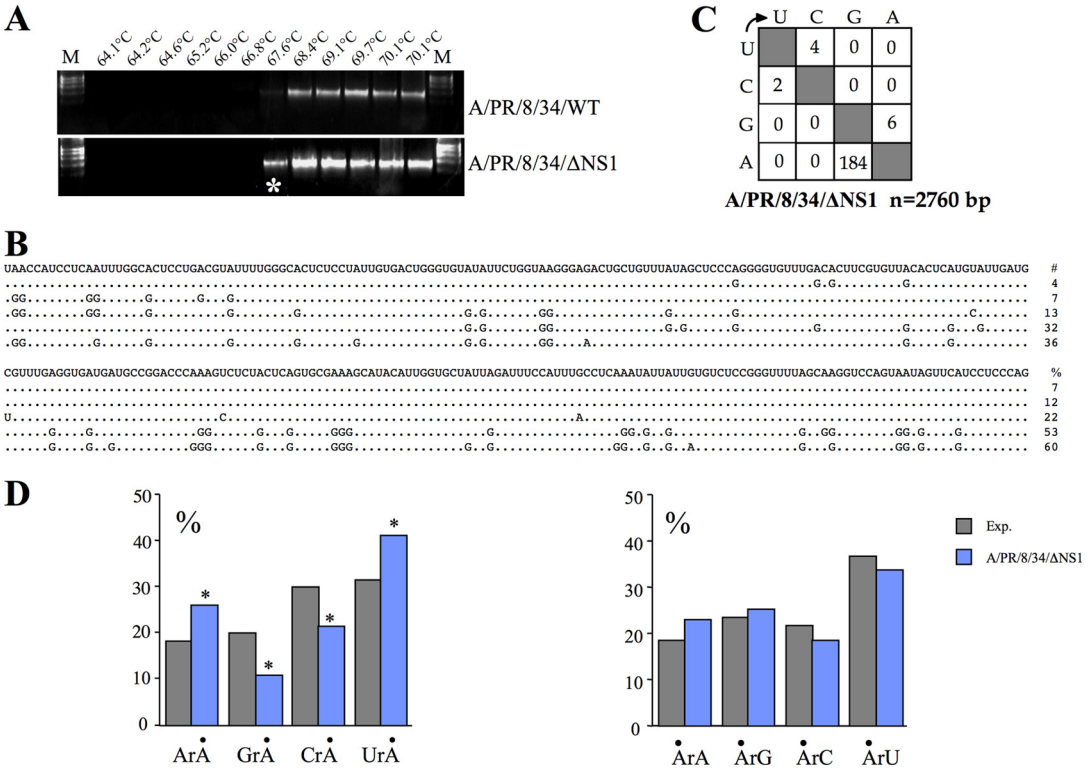

FIG. 3. ADAR editing of A/PR/8/34ΔNS1 H1N1 influenza virus A genomes. (A) 3DI-PCR of A/PR/8/34 and A/PR/8/34ΔNS1 influenza virus A H1N1 genomes. Asterisks indicate the PCR products cloned and sequenced. The size of the 3DI-PCR fragment is 324 bp. (B) A selection of hypermutated A/PR/8/34ΔNS1 influenza virus genomes. (C) Mutation matrix of hyperedited A/PR/8/34ΔNS1 influenza virus sequences. The number below the matrix refers to the number of bases sequenced. (D) Dinucleotide analysis in 5′ and 3′ of edited A/PR/8/34ΔNS1 influenza virus genomes. A χ^2 analysis indicates dinucleotide frequencies deviating significantly from the expected values (∗, $P < 0.05$).

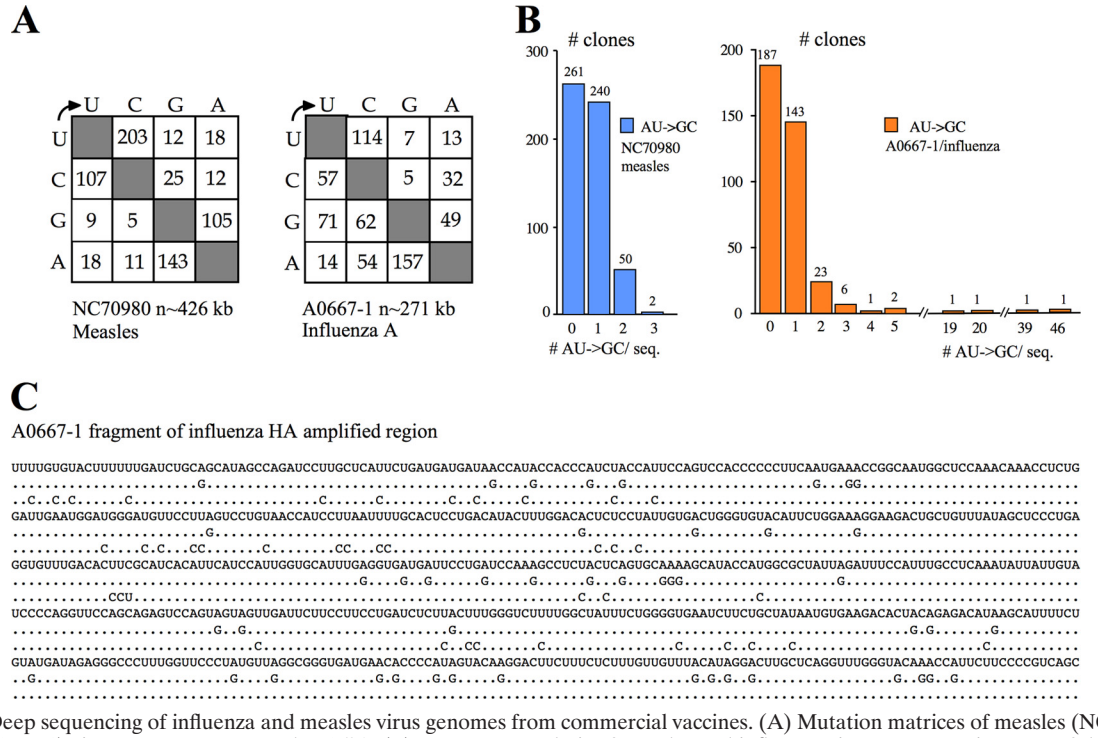

FIG. 4. Deep sequencing of influenza and measles virus genomes from commercial vaccines. (A) Mutation matrices of measles (NC70980) and influenza (A0667-1) virus genomes recovered at 95°C. (B) Frequency analysis of measles and influenza virus sequences in terms of the number of mutations per clone. (C) Hyperedited influenza virus A genome and antigenome sequences shown as differences with respect to the reference sequence. Only part of the 741-bp amplified fragment is shown.

For influenza virus, the primers were FVNC (5′ YGCYCCC AYAAYGGGAAAAGCAG) and RVNC (5′ TTGRRTTCTT TGCCCRCRGCTGTG) (770 bp). For measles virus, they were FMV (5′ CTACGACTTCGACAAGTCGGCATG) and RMV (5′ TGAACCCGAGTTGTGCATGGAGAG) (741 bp). For both viruses, the PCR parameters were 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C. We sequenced 553 and 366 measles and influenza virus clones representing ~700 kb.

For the measles virus vaccine, there were no *bona fide* A→G hypermutated genomes (Fig. 4A), even though the sequences harbored numerous AU→GC transitions (Fig. 4B). In contrast, four influenza virus sequences with between 19 and 46 mutations each were identified (Fig. 4B), indicating a hypermutant frequency of ~1%. Surprisingly, one of these was hyperedited on the plus strand or antigenome (Fig. 4C). As it is monotonously substituted, it presumably reflects editing of the antigenome rather than a highly folded genome sequence that would be expected to yield both A→G and U→C substitutions. In turn, this indicates that ADAR can restrict both the viral genome and the replication template of influenza virus. A similar observation was made for Rift Valley fever virus (28).

The findings for influenza virus A widen the field of RNA viruses that can be restricted by ADAR. Is there any function left in a hyperedited viral sequence? While such genomes must be functionally dead and marked out for degradation by inosine-specific ribonucleases (26), as well as other RNases, the finding of hyperedited flu virus genomes at a reasonable frequency, ~1%, suggests that inosine might represent a novel danger signal. It is well known that double-stranded poly(I · C) is a better inducer of type I IFNs than double-stranded poly(G · C) (11). Although this is a nonphysiological substrate, it might reflect in an exaggerated form the action of ADAR-edited double-stranded RNA (dsRNA), as a full-length dsRNA duplex of folded ssRNA picked up by a either a macrophage or dendritic cell. Whether this contributes in any way to the attenuated phenotype of some live viral vaccines is an open question.

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